MAJOR ARTICLE

Escape from Immune Surveillance by *Capnocytophaga canimorsus*

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***Capnocytophaga canimorsus*, a commensal bacterium from dogs' mouths, can cause septicemia or meningitis in humans through bites or scratches. Here, we describe and characterize the inflammatory response of human and mouse macrophages on *C. canimorsus* infection. Macrophages infected with 10 different strains failed to release tumor necrosis factor (TNF)–α and interleukin (IL)–1α. Macrophages infected with live and heat-killed (HK) *C. canimorsus* 5 (*Cc*5), a strain isolated from a patient with fatal septicemia, did not release IL-6, IL-8, interferon-γ, macrophage inflammatory protein–1β, and nitric oxide (NO). This absence of a proinflammatory response was characterized by the inability of Toll-like receptor (TLR) 4 to respond to *Cc*5. Moreover, live but not HK *Cc*5 blocked the release of TNF-α and NO induced by HK *Yersinia enterocolitica*. In addition, live *Cc*5 down-regulated the expression of TLR4 and dephosphorylated p38 mitogen-activated protein kinase. These results highlight passive and active mechanisms of immune evasion by *C. canimorsus*, which may explain its capacity to escape from the host immune system.**

Millions of people are bitten by dogs every year, but only 5%–10% of dog bites become infected. The overall mortality of such infections is around 6 deaths out of 100 million population annually in the United States [1, 2]. However, since 1976, there have been numerous case reports describing sepsis or meningitis after dog bites. Bacteria isolated from these dramatic cases were found to belong to a new species, which was later called *Capnocytophaga canimorsus* [3].

The genus *Capnocytophaga* contains 7 species, all of them encountered in the oral cavity of humans or domestic animals. But *C. canimorsus,* commonly found in dogs, is the only one associated with severe human infections [4]. The genus *Capnocytophaga* belongs to the class of Flavobacteria. Flavobacteria in turn belong to the Bacteroidetes phylum, which includes various species of *Bacteroides* and *Porphyromonas gingivalis* [5, 6]. In the bacterial evolutionary tree, the Bacteroidetes phylum is very remote from proteobacteria and the common human pathogens. Although substantial knowledge on the metabolism and genetics of *Bacteroides* species exists, much less is known about Flavobacteria and especially about *Capnocytophaga* species.

More than 160 cases of severe infections with *C. canimorsus* have been reported in the literature. Patients infected with *C. canimorsus* have been bitten, scratched, or sometimes simply licked by a dog or occasionally by a cat. Symptoms usually appear after 2–3 days, and patients are generally admitted to the hospital several days after the exposure, with symptoms of sepsis or meningitis. Mortality is as high as 30% for septicemia, but it is only around 5% for meningitis [7]. Many of these infections involve patients who have had a splenectomy, are alcoholic, or are immunocompromised, but a significant number involve healthy people with no obvious risk factors [8–11]. *C. canimorsus* is thus an emerging pathogen, and cannot be considered simply as an opportunistic bacterium. Little is known about the pathogenesis of infection with *C. canimorsus.* It has been reported to multiply in J774.1 mouse macrophages and to be cytotoxic, presumably by secreting a toxin [12]. More studies have been devoted to *C. ochracea,*

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which is an agent of periodontal disease and has been reported to degrade lactoferrin [13], IgG [14], and IgA [15] and to produce an immunosuppressive factor [16]. In addition, the lipopolysaccharide (LPS) from *C. ochracea* has been shown to be an antagonist for human Toll-like receptor (TLR) 4 [17].

The overall clinical evolution of *C. canimorsus* infections suggests that the bacterium elicits little inflammation, at least in the early stages of the infection, which would allow time for multiplication, up to a stage at which it causes general sepsis and a deadly shock. In some cases, the patient even died of a secondary aspergillosis, suggesting that *C. canimorsus* might have induced some immunodeficiency (J. B. le Polain, personal communication). In this article, we test this hypothesis, and we show that indeed *C. canimorsus* does not elicit inflammation and that at least 2 different mechanisms contribute to this effect.

MATERIALS AND METHODS

Bacterial strains and identification. The strains of *C. canimorsus* are listed in table 1. The identity of the clinical isolates was confirmed by 16S rRNA sequencing using primers 27F and 1100R [20]. Alignment analysis of 16S rRNA was performed using T-COFFEE (available at: http://www.EMBnet.org)[21]. Wild-type *(wt) Yersinia enterocolitica* E40 (pYV40) and multieffector knockout mutant E40 (pIML421), commonly called ΔHOPEMT, were used as control bacteria [22].

Bacterial growth condition. *C. canimorsus* was routinely grown on Heart Infusion Agar (Difco) supplemented with 5% sheep blood (Oxoid) for 2 days at 37°C in an incubator containing 5% CO_2. *Y. enterocolitica* was pregrown overnight with continuous shaking (120 rpm) in brain-heart infusion at 22°C. Before infection, the type III secretion system of *Y. enterocolitica* was induced as described elsewhere [23].

Cell culture and infection. Murine monocyte-macrophage J774.1 cells (ATCC TIB-67), human monocytic THP-1 cells (ATCC TIB-20), and HEK293 cells (ATCC CRL-1573) were cultured as recommended by the American Type Culture Collection. Isolation and maintenance of bone marrow–derived macrophages (BMDMs; provided by B. Combaluzier) from C57/BI6 mice has been described elsewhere [24]. Human monocytes were isolated from peripheral-blood mononuclear cells using Ficoll-Paque density gradient centrifugation and were further purified using anti-CD14 microbeads (Miltenyi Biotec). Unless otherwise indicated, cells were seeded in medium without antibiotics at a density of 10^5 cells/cm^2 15 h before infection. Infection was performed at 37°C at an MOI of 20. When specified, bacteria were killed by the addition of ceftazidime (final concentration of 10 μg/mL). For priming of J774.1 cells, cells were pretreated with interferon (IFN)–γ (500 U/mL; BD Pharmingen) for 48 h before infections. Increased respiratory burst in IFN-γ–primed J774.1 cells was measured using 3′-(*p*-aminophenyl) fluorescein, as recommended by the manufacturer (Molecular Probes).

Video microscopy. J774.1 cells were placed under a Leica DMIRE2 microscope equipped with a thermostated (37°C) and CO_2-equilibrated chamber. Three-hour video sequences were recorded using a digital camera (Hamamatsu Photonics) and OpenLab software (version 3.1.2). Sequences were converted to QuickTime format (Apple).

Cytokine assay. Cell-free supernatants were assayed for cytokine and chemokine production using commercial ELISA kits (BD Pharmingen) or the Bio-Plex mouse and human cytokine multiarray system (Bio-Rad Lab).

Nitric oxide (NO) assay. NO production was estimated as the amount of nitrite released in the culture medium, by use of modified Griess reagent (Sigma).

Lactate dehydrogenase (LDH) release assay. LDH release determinations were performed using the CytoTox 96 cytotoxicity assay kit (Promega).

NF-κB assay. Human TLR2, TLR4, TLR5, MD-2, and CD14 expression plasmids (provided by M. F. Smith, C. J. Kirschning, and K. Miyake) have been described elsewhere [25]. Ligands for TLRs were obtained from Invivogen. HEK293 cells were transfected in 12-well plates using Fugene 6 (Roche). Each transfection contained 100 ng of TLR, 100 ng of MD-2, 100 ng of CD14, 500 ng of pELAM-Luc, 100 ng of pCMV–β-galactosidase, and 3 μL of Fugene 6. Transfections were cultured for 24 h, and cells were stimulated as indicated for an additional 24 h. NF-κB activity was determined by measuring the luciferase activity present in cell extracts. Luciferase values were normalized for differences in transfection efficiency on the basis of β-galactosidase activity in the same extracts and were expressed as fold induction values relative to the unstimulated empty vector control.

Immunoblotting. For each experimental condition, 3×10^6 J774.1 cells were harvested by centrifugation at 20,000 *g* for 5 min and resuspended in 100 μL of cell lysis buffer (20 mmol/L Tris, 100 mmol/L NaCl, 0.2% [vol/vol] Triton X-100, and 10 mmol/L EDTA [pH 7.4]). Lysates were centrifuged at 20,000 *g* for 5 min and diluted in 5× SDS/PAGE sample buffer. Samples were boiled for 5 min, and proteins were separated on 12% SDS/PAGE gel. Anti-p38 mitogen-activated protein kinase (MAPK) and anti–phospho-p38 MAPK were purchased from Cell Signaling Technology.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted using the RNeasy Kit (Qiagen). RT of RNA was performed with 1 μg of RNA, RT buffer, 0.4 mmol/L each dNTP, 40 U of RNaseOUT (Invitrogen), 8 mmol/L DTT, 500 ng of oligo(dT) primer, and 10 U of Superscript II reverse transcriptase (Invitrogen) at 42°C for 1 h. PCR primer sequences are listed in table 2. The reaction mixtures were subjected to 30 (β-actin, TLR2, and TLR4), 45

Table 1. ***Capnocytophaga canimorsus (Cc)*** **strains used in this study.**

Strain	Collection no.	Identity,[a] %	Biological origin	Year of isolation	History and geographical origin	Reference
*Cc*2	…	96.2	Human fatal septicemia after dog bite	1989	G. Wauters & M. Delmée, Univ. Clinics St. Luc, Brussels, Belgium	[9]
*Cc*3	…	99.0	Human septicemia	1990	G. Wauters & M. Delmée ← St. Jan Hospital, Brugge, Belgium	[18]
*Cc*5	…	100	Human fatal septicemia after dog bite	1995	G. Wauters & M. Delmée ← Clinic of Libramont, Libramont, Belgium	…
*Cc*7	…	99.6	Human septicemia	1998	G. Wauters & M. Delmée ← KUL, Leuven, Belgium	…
*Cc*9	BCCM/LMG 11510, CCUG 12569, CDC A3626	100	Human septicemia	1965	BCCM/LMG ← CCUG ← R. Weaver, CDC, Atlanta, Georgia ← Virginia	[19]
*Cc*10	BCCM/LMG 11541, CCUG 17234, ATCC 35978	100	Human septicemia after dog bite	…	BCCM/LMG ← MCCM ← ATCC ← R. Weaver, CDC, Atlanta, Georgia ← California Health Dept. (324-1-74)	[19]
*Cc*11	BCCM/LMG11551, MCCM 01373	99.1	Human septicemia	…	BCCM/LMG ← MCCM ← A. von Graevenitz, Univ. Zürich, Zürich, Switzerland	…
*Cc*12	ATCC 35979, CDC 7120	100	Human septicemia after dog bite	…	ATCC ← R. Weaver, CDC, Atlanta, Georgia ← California Health Dept. ← San Antonio Community Hospital, San Antonio, Texas	…
*Cc*13	…	99.4	Healthy dog's (Jackie) saliva	2005	Direct isolation, Basel, Switzerland	Present study
*Cc*14	…	100	Healthy dog's (Pouchka) saliva	2005	Direct isolation, Basel, Switzerland	Present study

NOTE. ATCC, American Type Culture Collection; BCCM/LMG, Belgian Co-ordinated Collection of Microorganisms; CCUG, Culture Collection University of Göteborg; CDC, Centers for Disease Control and Prevention; KUL, Katholieke Universiteit Leuven; MCCM, Medical Culture Collection Marburg.

[a] Determined by comparing the 16S rRNA sequence of each strain to a reference strain (*Cc*12; ATCC 35979). Minimal length of 16S rRNA sequence analyzed is 300 bp.

Table 2. Sequences of oligonucleotides used in reverse-transcription polymerase chain reaction.

	Sequences (5′→3′)	
Oligonucleotide	Forward	Reverse
TLR2	cagcttaaagggcgggtcagag	tggagacgccagctctggctca
TLR4	agtgggtcaaggaacagaagca	ctttaccagctcatttctcac
MyD88	ggatgcctggcaggggcgct	gctctgtagataatcgtcag
IRAK-M	gcttcggaatcgtgatc	cgtgggtcttaacttggcc
SOCS-1	ttctggaaccacgtgggag	caggtcctggccccagacac
β-actin	taaaacgcagctcagtaacagtccg	tggaatcctgtggcatccatgaaac

NOTE. IRAK, interleukin-1 receptor–associated kinase; MyD, myeloid differentiation factor; SOCS, suppressor of cytokine signaling; TLR, Toll-like receptor.

(interleukin [IL]–1 receptor–associated kinase M [IRAK-M]), and 35 (myeloid differentiation factor 88 [MyD88] and suppressor of cytokine signaling 1 [SOCS-1]) cycles of amplication.

Statistical analysis. Results are median or mean ± SD values. Quantitative data were compared by use of the Mann-Whitney *U* test. Differences were considered to be significant when $P < .05$.

RESULTS

Replication and survival of* C. canimorsus *in the presence of J774.1 mouse macrophages. Macrophages are one of the primary mammalian defense lines against bacterial pathogens. They not only recognize, engulf, and kill microorganisms, but they also mobilize the antimicrobial host defense by secreting chemokines and cytokines. We first investigated whether *C. canimorsus* can survive in the presence of mouse macrophages. We analyzed *C. canimorsus*–infected J774.1 macrophages by video microscopy and observed that bacteria interacting with macrophages were not internalized (figure 1*A*). In agreement with these observations, viable count experiments showed that *C. canimorsus* survive and multiply in the presence of J774.1 cells (figure 1*B*). Furthermore, we found that the presence of macrophages in the cell culture medium enhanced bacterial growth by ~100-fold at 24 h after infection. Addition of cytochalasin D had little effect on the bacterial growth rate, confirming that there was an insignificant level of phagocytosis. We also monitored cytotoxicity for macrophages by measuring LDH release. As controls, we included *wt* (E40 *wt*) and multieffector knockout (ΔHOPEMT) *Y. enterocolitica* strains. Interestingly, the growth of *C. canimorsus* did not induce cytotoxicity (figure 1*C*). Macrophages infected with *C. canimorsus* remained unaltered, adherent, and viable, as examined by trypan blue dye exclusion (data not shown). As expected, cytotoxicity was observed by *wt Y. enterocolitica* [26] and by ΔHOPEMT *Y. enterocolitica* [27]. Hence, *C. canimorsus* seems to replicate in the presence of macrophages without inducing cytotoxicity.

Absence of potent proinflammatory response in mouse macrophages. To determine whether *C. canimorsus* infection induces secretion of proinflammatory cytokines, J774.1 cells were infected with 10 different strains of *C. canimorsus* at a 20:1 bacteria/macrophage ratio, and culture supernatants from 24 h after infection were analyzed for the release of tumor necrosis factor (TNF)–α and IL-1α. As shown in figure 2*A*, *C. canimorsus* induced only negligible amounts of TNF-α and IL-1α, similar to those of mock-infected control cells and to those induced by E40 *wt,* which prevents inflammation by injecting YopP [28], YopE, and YopT [29] into cells. As expected, the multieffector knockout *Y. enterocolitica* ΔHOPEMT strain effectively induced secretion of TNF-α and IL-1α [30]. Further analysis was pursued with *C. canimorsus* 5 (*Cc*5; provided by M. Delmée, University of Louvain, Belgium), a strain isolated from a patient with fatal septicemia (table 1). Production of proinflammatory IL-6 and anti-inflammatory IL-10 by cells infected with both live and heat-killed (HK) *Cc*5 was only marginal, compared with that by mock-infected control cells (figure 2*A*). To test whether this lack of cytokine release was due to a low dose of bacteria, macrophages were exposed to *Cc*5 or ΔHOPEMT for 2 h at various bacteria/macrophage ratios, and supernatants from 24-h cultures were tested for TNF-α production. Although we observed some dose dependency in the response of macrophages to *Cc*5, the amount of TNF-α released was significantly less than that release by ΔHOPEMT-infected cells.

Priming of macrophages with IFN-γ has been shown to increase cellular responsiveness to inflammatory stimuli [31, 32]. To test whether primed macrophages exhibit an enhanced response to *C. canimorsus,* J774.1 cells were prestimulated with IFN-γ for 48 h, infected, and analyzed for cytokines, as described above. Surprisingly, we could not detect a significant release of IL-1α, IL-6, or TNF-α in activated J774.1 cells infected with either live or HK *Cc*5 (figure 2*B*).

We next examined the production of IL-6, IFN-γ, and TNF-α in *C. canimorsus*–infected BMDMs 24 h after infection. We

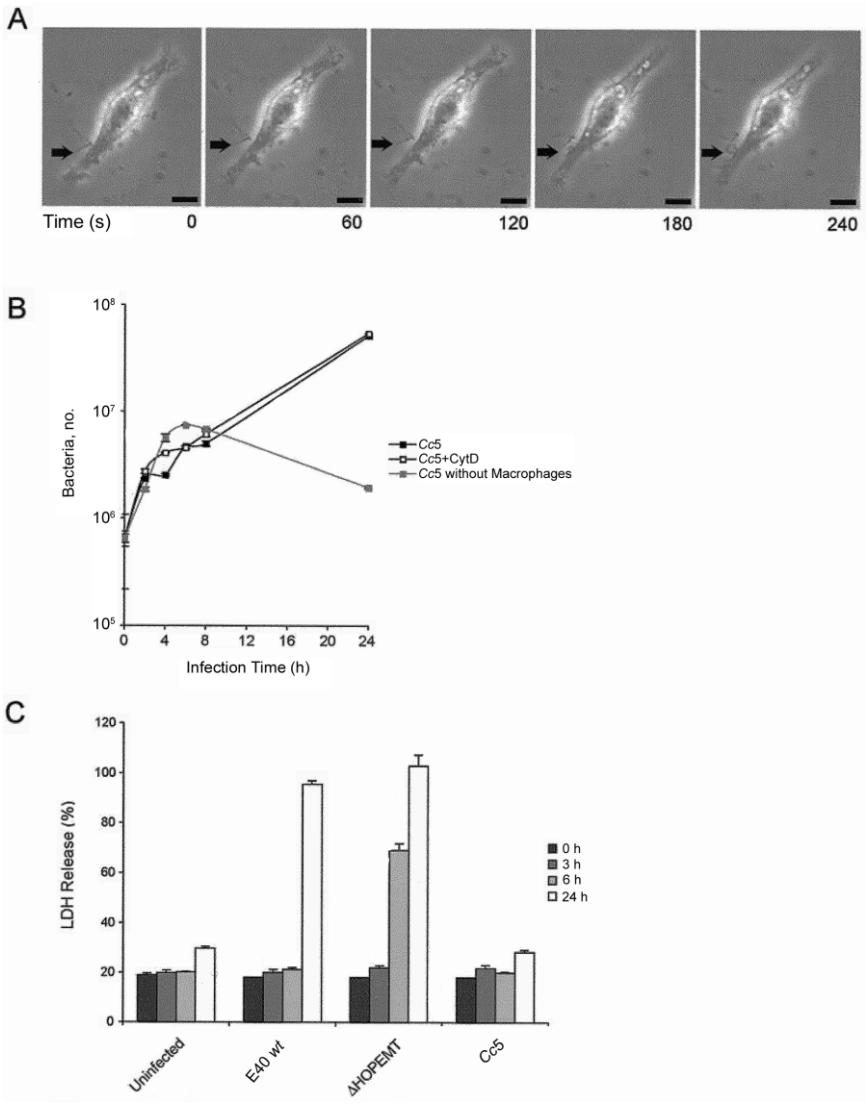

Figure 1. Interaction, survival, and multiplication of *Capnocytophaga canimorsus* in the presence of mouse macrophages. *A,* J774.1 cells, infected with *C. canimorsus* 5 (*Cc*5) (MOI of 20). After infection, 3-h video sequences were recorded. Only 1 sequence of representative images is shown. Images were acquired at intervals of 60 s. Arrows indicate the position of the same bacterium during 4 min. Bar, 2 μm. *B,* J774.1 cells, infected with *Cc*5 (MOI of 20). Cytochalasin D (CytD; 5 μg/mL) was added 30 min before infection and was retained during the infection. The total no. of bacteria was determined by serial dilution and agar plating of the culture medium collected at the indicated time points. Results are representative of 3 independent experiments. *C,* J774.1 cells, infected with various strains (MOI of 20) for the indicated times. Culture supernatants were collected, and the level of lactate dehydrogenase (LDH) was measured using a CytoTox 96 assay kit. Error bars represent the SD values obtained from triplicate samples. *wt,* wild type.

observed relatively moderate levels of IL-6, IFN-γ, and TNF-α on infection with either live or HK *Cc*5 (figure 2*C*). Although levels of cytokines released on infection with *Cc*5 were somewhat higher than those released by infected J774.1 cells, they were significantly lower than those released by BMDMs infected with HK *Y. enterocolitica.*

No induction of a proinflammatory response in human monocytes by* C. canimorsus *infection. To avoid any bias due to host species specificity, human monocytic THP-1 cells were infected, and culture supernatants from 6 h after infection were analyzed for the release of IL-1β, IL-6, IL-8, IFN-γ, TNF-α, and macrophage inflammatory protein–1β. Consistent with the weak immune responses of mouse macrophages, neither live nor HK *Cc*5 induced detectable proinflammatory cytokine and chemokine production (figure 3*A*). Infection with live or HK *Cc*5 of human monocytes isolated from peripheral blood also resulted in low levels of IL-8 and TNF-α (figure 3*B*). These results together demonstrate that not only mouse but also human macrophages fail to trigger robust immune response to *C. canimorsus* infection.

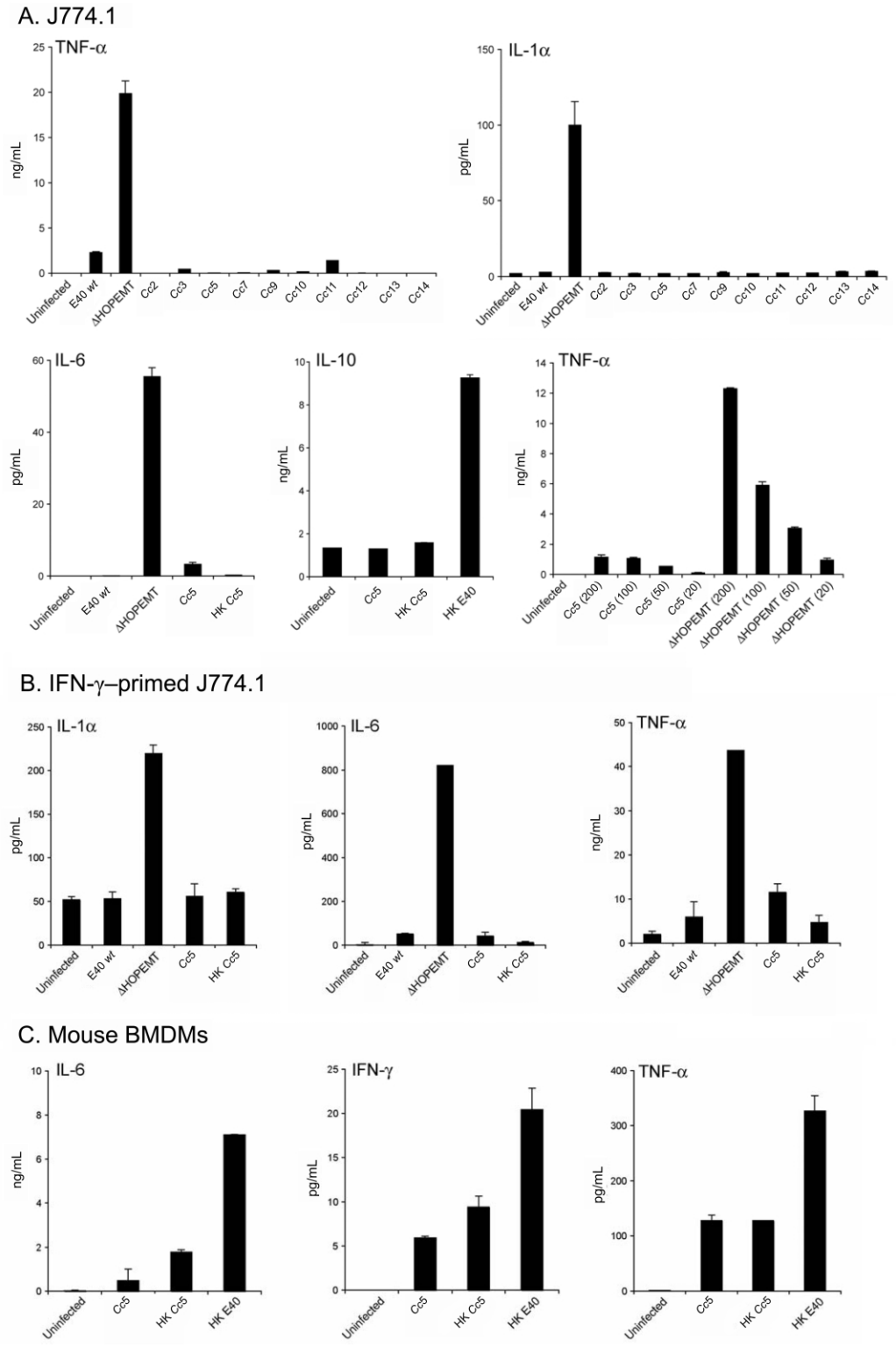

Figure 2. Absence of tumor necrosis factor (TNF)–α, interleukin (IL)–1α, IL-6, and IL-10 release by mouse macrophages infected with *Capnocytophaga canimorsus*. *A,* J774.1 cells, either mock infected or infected (MOI of 20) for 24 h with the indicated strains. To determine the dose response of TNF-α release, the indicated strains were used to infect macrophages at the indicated MOIs. Bacteria were killed with ceftazidime after 2 h, and supernatants from 24 h after infection were analyzed for TNF-α production. *B,* J774.1 cells, prestimulated with interferon (IFN)–γ (500 U/mL) for 48 h and either mock-infected or infected (MOI of 20) for 24 h with the indicated strains. *C,* Bone marrow–derived macrophages (BMDMs), infected with the indicated strains (MOI of 20). Cell culture supernatants were collected 24 h after infection, and levels of cytokine production were analyzed by ELISA. The results are representative of 5 independent experiments. Error bars represent the SD values. HK, heat killed; *wt,* wild type.

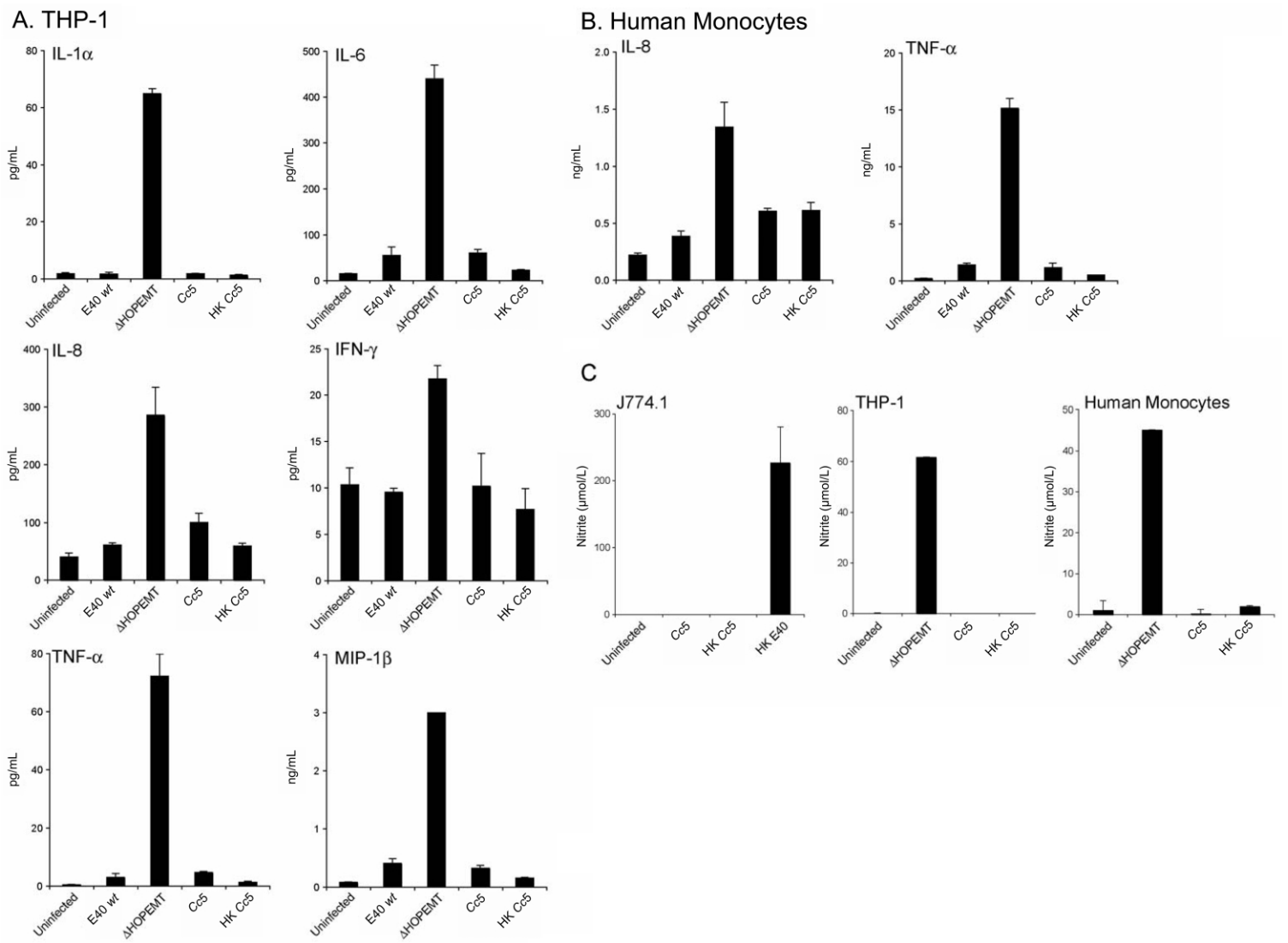

Figure 3. Cytokine, chemokine, and nitric oxide (NO) production during *Capnocytophaga canimorsus* infection. *A,* THP-1 cells, either mock infected or infected (MOI of 20) for 6 h with the indicated strains. Levels of interleukin (IL)–1β, IL-6, IL-8, interferon (IFN)–γ, tumor necrosis factor (TNF)–α, and macrophage inflammatory protein (MIP)–1β were analyzed by ELISA. *B,* Human monocytes, either mock-infected or infected (MOI of 20) for 5 h with the indicated strains. Levels of IL-8 and TNF-α were measured by ELISA. *C,* J774.1 and THP-1 cells and human monocytes, either mock infected or infected (MOI of 20) with the indicated strains for 24 h, 6 h, and 5 h, respectively. Cell culture supernatants were collected, and the level of NO was determined using Griess reagent. Data are the mean for triplicates from 5 independent experiments. Error bars represent the SD values. HK, heat killed; *wt,* wild type.

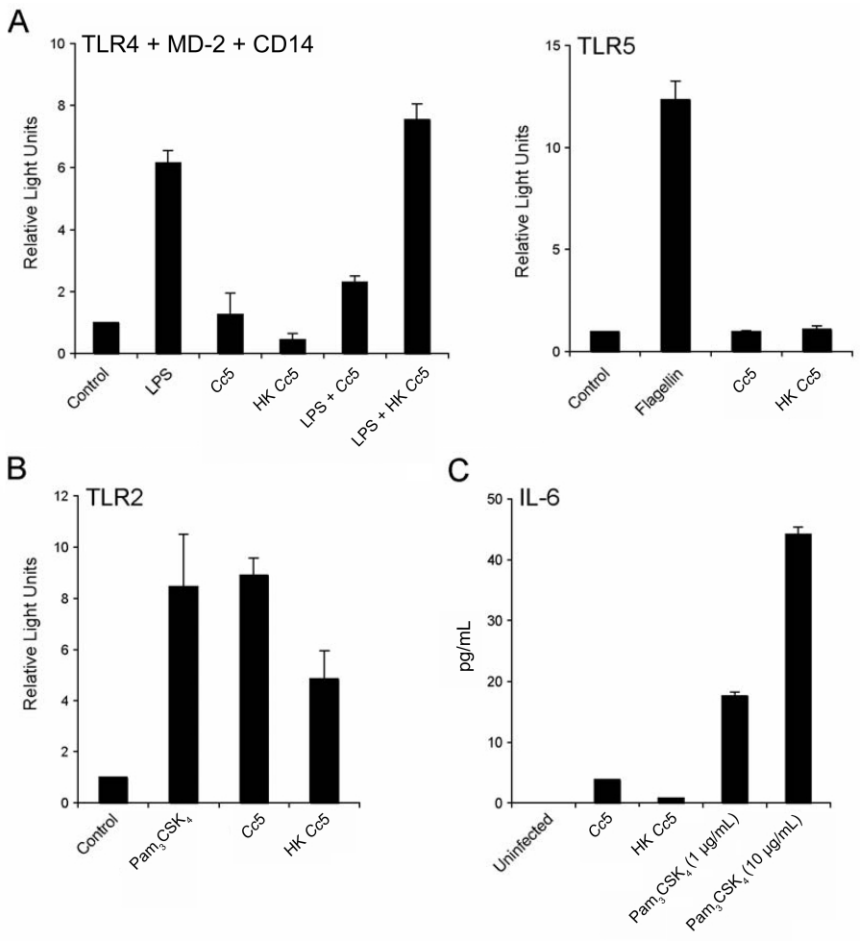

Figure 4. *Capnocytophaga canimorsus* 5 (*Cc*5) interaction with Toll-like receptors (TLRs). *Cc*5 does not interact TLR4 or TLR5 but does interact with TLR2. HEK293 cells were transiently transfected with NF-κB–Luc, pEF6–MD-2, pRcRSV-CD14, and either pCDNA3.1 or expression plasmids for TLR4 and TLR5 *(A)* or TLR2 *(B)*. Twenty-four hours after transfection, cultures were stimulated with 1 μg/mL *Escherichia coli* lipopolysaccharide (LPS), 100 ng/mL Pam_3CSK_4, 1 μg/mL flagellin, or live or heat-killed (HK) *Cc*5 (MOI of 20). For inhibition of TLR4 activation by *Cc*5, live or HK *Cc*5 (MOI of 20) and LPS were added simultaneously. Luciferase activities were determined 24 h later and were normalized to cotransfected β-galactosidase activity. *C,* J774.1 cells, infected with the indicated strains (MOI of 20) or Pam_3CSK_4 (1 or 10 μg/mL) for 24 h. Culture supernatants were analyzed for interleukin (IL)–6 release by ELISA. Results are mean ± SD values of triplicate transfections and are representative of 3 experiments performed.

Lack of detectable NO production by* C. canimorsus *in mouse and human macrophages. NO is one of the primary mediators of the host cell defense against many intracellular and extracellular bacteria. We, therefore, assessed NO production by *C. canimorsus*–infected J774.1, THP-1, and human monocytes. Cells were infected as described above, and the amount of NO was measured as nitrite production. Although J774.1, THP-1, and human monocytes were capable of NO production in response to HK or ΔHOPEMT *Y. enterocolitica,* infection with live or HK *Cc*5 did not result in any detectable NO production (figure 3*C*).

Lack of interaction of* Cc*5 with TLR4 and TLR5. TLRs are a family of cell surface molecules that participate in innate immune response by recognizing pathogen-associated molecular patterns. Recognition and subsequent stimulation of TLRs trigger signaling events that result in the production of proinflammatory cytokines [33]. We next investigated whether TLRs are capable of recognizing *C. canimorsus.* To determine which, if any, TLRs may play a role in the response of macrophages to *C. canimorsus,* we examined the *C. canimorsus*–induced NF-κB response of HEK293 cells transfected with TLR2, TLR4, or TLR5. In the transfection experiment shown in figure 4, HEK293 cells were cotransfected with the NF-κB–Luc reporter, pCMV–β-galactosidase plasmids, and either empty pcDNA3.1 vector or expression plasmids for TLR2, TLR4 (with CD14 and MD-2 expression plasmids), or TLR5. Cultures were stimulated with either known purified TLR ligands (Pam_3CSK_4 for TLR2, *Escherichia coli* LPS for TLR4, and *Salmonella thyphimurium* flagellin for TLR5) and live or HK *Cc*5 for 24 h and assayed for luciferase activity.

Cells transfected with TLR4 or TLR5 were unable to activate NF-κB in response to *C. canimorsus* infection (figure 4*A*). The

transfected TLR4 and TLR5 were indeed functional, as demonstrated by the ability of the cells to activate NF-κB in response to *E. coli* LPS or flagellin, respectively. We next tested whether *C. canimorsus* can act as an antagonist of TLR4. To this end, we stimulated transfected cells simultaneously with *E. coli* LPS and live or HK *Cc*5. Live but not HK *Cc*5 prevented *E. coli* LPS–induced TLR4 stimulation (figure 4*A*). This suggests that *C. canimorsus* can indeed inhibit NF-κB activation induced by TLR4, but this abrogation is not mediated by the antagonistic action of its LPS.

TLR2-transfected HEK293 cells were capable of activating NF-κB in response to live or HK *Cc*5 (figure 4*B*). To compare the cytokine-inducing capacity of *Cc*5 with that of a TLR2 agonist (Pam_3CSK_4), we infected J774.1 cells with either live or HK *Cc*5 and Pam_3CSK_4 (1 or 10 μg/mL) for 24 h and analyzed the release of IL-6. As shown in figure 4*B,* the level of IL-6 release was significantly lower in J774.1 cells infected with either *Cc*5 or HK *Cc*5 than with Pam3CSK4. Thus, whereas *Cc*5 (or HK *Cc*5) could lead to NF-κB activation via TLR2, this activation did not lead to a potent inflammatory response by macrophages.

***Inhibition by* Cc*5 of proinflammatory response induced by HK* Y. enterocolitica.** To further analyze the antagonistic action of *Cc*5 on TLR4 activation, we examined whether *Cc*5 can block the proinflammatory response induced by HK *Y. enterocolitica.* J774.1 cells were incubated with live or HK *Cc*5 (MOI of 20) in the presence or absence of HK *Y. enterocolitica*, and the supernatants from 24 h after infection were tested for TNF-α and NO release. The results shown in figure 5*A* clearly demonstrate that there was no TNF-α or NO in the culture supernatants when live but not HK *Cc*5 were added together with HK *Y. enterocolitica.* This inhibitory effect could also be observed when J774.1 cells were preinfected with live *Cc*5 for 24 h and then stimulated with HK *Y. enterocolitica.* Live *Cc*5, however, could not inhibit TNF-α and NO release if J774.1 cells were prestimulated with HK *Y. enterocolitica* (data not shown). Addition of cytochalasin D (5 μg/mL) during infection had no effect on the ability of *Cc*5 to suppress TNF-α and NO release, showing that internalization of *C. canimorsus* is not necessary for this action (figure 5*B*). Release of several other cytokines and chemokines, such as IL-3, IL-5, IL-6, IL-17, and RANTES (data not shown), was also inhibited by live *Cc*5, suggesting that the antagonistic effect of live *Cc*5 is global.

Regulation of TLR4 and p38 MAPK by* Cc*5. To determine whether *Cc*5 can modulate the expression level of key molecules involved in the production of a proinflammatory response, we analyzed the mRNA level of TLR2, TLR4, MyD88, IRAK-M, and SOCS-1 in J774.1 cells infected with live or HK *Cc*5 in the presence or absence of HK *Y. enterocolitica* for 24 h by RT-PCR (figure 5*C*). We found no significant change in the level of TLR2, MyD88, IRAK-M, and SOCS-1 on infection with either live or HK *Cc*5, suggesting that *Cc*5 does not directly regulate the expression of negative regulators, such as IRAK-M and SOCS-1, nor of MyD88 and TLR2. In contrast, mRNA expression of TLR4 infected with live *C. canimorsus* was reduced. Down-regulation in TLR4 mRNA level was not the result of *C. canimorsus* LPS–induced tolerance, because HK *Cc*5 did not have an effect. Finally, we assessed the activation of p38 MAPK, one of the prominent targets of the TLR signaling cascade. The activation state of p38 MAPK was analyzed using antibody specific for its phosphorylated form. Infection of J774.1 cells with live *Cc*5 for 24 h resulted in complete dephosphorylation of p38, whereas HK *Cc*5 had no effect. *Cc*5 infection did not alter the level of total p38 (figure 5*D*). It is interesting to note that suppression of p38 phosphorylation by live *Cc*5 coincided with the inhibition of inflammatory responses as well as the down-regulation of TLR4.

DISCUSSION

The clinical overview of *C. canimorsus* infections hints at the ability of the bacterium to avoid the immune system, at least in the early stages of the infection. To this end, we characterized the immune response from mouse and human macrophages on *C. canimorsus* infection. We found that *C. canimorsus* does not activate signals leading to the release of proinflammatory cytokines, chemokines, and NO. This confirms and extends a previous report that normal whole blood produces lower levels of IL-1β and IL-6 in response to *C. canimorsus* than in response to other gram-negative bacteria [34]. Both mouse and human macrophages were unable to produce potent proinflammatory responses on infection with either live or HK *C. canimorsus.* Our data thus suggest relatively silent entry of *C. canimorsus* into the host, where it replicates and survives in the presence of macrophages. We did not observe any cytotoxicity by the 10 strains of *C. canimorsus* for macrophages. This result is at odds with the data from Fischer et al. [12], especially because the strain used in this previous study was included in our experiments [12]. At this time, we have no explanation for this discrepancy.

Why are macrophages unable to mediate proinflammatory responses against *C. canimorsus?* We found that one of the immune sensors of invading pathogens, TLR4, is unable to activate NF-κB on encountering either live or HK *C. canimorsus.* This suggests that the lipid A moiety of *C. canimorsus* LPS may be different from that of the enteric bacteria, for example, in its acylation pattern [35]. Further investigation on the structure of *C. canimorsus* LPS is under way, to determine its structure and to characterize its endotoxic activity. Although *C. canimorsus* does not react with TLR4, it interacts with TLR2. Activation of TLR2 by *C. canimorsus,* however, does not seem to result in a potent proinflammatory response. Further investigation of the functional role of TLR2 activation, as well as identification of the TLR2 ligand from *C. canimorsus,* is needed.

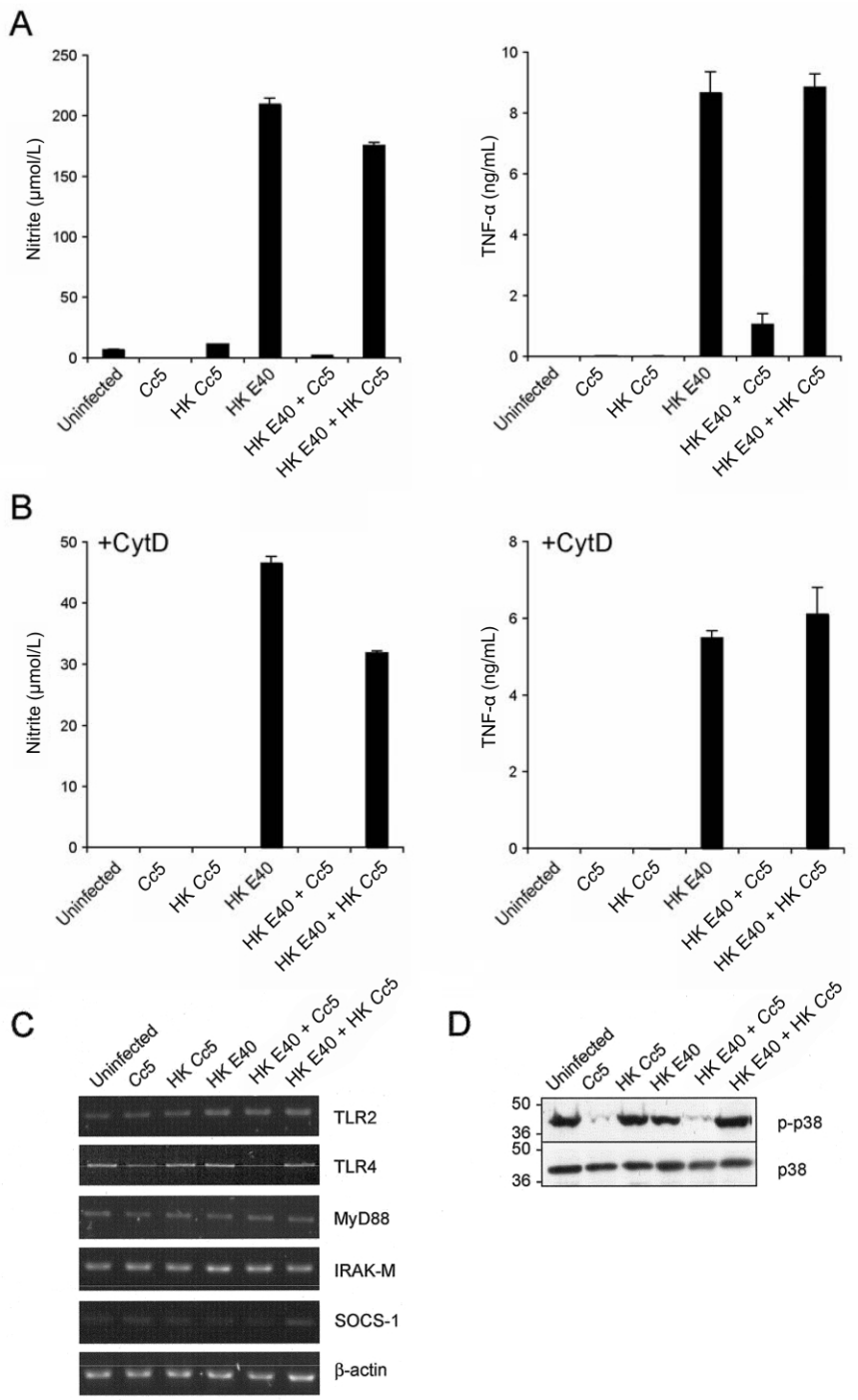

Figure 5. Inhibition of heat-killed (HK) *Yersinia enterocolitica*–induced proinflammatory response by *Capnocytophaga canimorsus* 5 (*Cc*5). *A,* J774.1 cells, infected with the indicated strains (MOI of 20) together with or without HK E40 (MOI of 20) for 24 h. Culture supernatants were analyzed for the release of nitric oxide and tumor necrosis factor (TNF)–α. *B,* J774.1 cells, infected as in panel A, in the presence of cytochalasin D (CytD) at 5 μg/mL. *C,* J774.1 cells, amplified by polymerase chain reaction (PCR). After infection of J774.1 cells with the indicated strains (MOI of 20) for 24 h, total RNA was extracted, reverse transcribed, and amplified by PCR. The PCR products were separated in 1.2% agarose gel. *D,* J774.1 cells, infected with the indicated strains (MOI of 20) for 24 h. After infection, cell lysates were prepared and separated on a 12% SDS-PAGE gel. Immunoblotting was performed using an antibody specific for the phosphorylated form of p38 (p-p38) and total p38. IRAK-M, interleukin-1 receptor–associated kinase M; MyD88, myeloid differentiation factor 88; SOCS-1, suppressor of cytokine signaling 1.

We further provide evidence that NO, TNF-α, and other proinflammatory cytokines are absent from the culture supernatant of macrophages when stimulated by HK *Y. enterocolitica* in the presence of live *C. canimorsus*. Live *Cc*5 could degrade and/or prevent the release of NO and cytokines produced by macrophages through interference with the proinflammatory-signaling cascade. We favor the second interpretation for 2 reasons. First, it would be unlikely that *Cc*5 would degrade NO and all tested cytokines. Second, infection of live *Cc*5 leads to complete dephosphorylation of p38 MAPK, whereas HK *Cc*5 does not. Down-regulation of p38 MAPK activation correlates well with its critical role in cytokine production, phagocytosis, and regulation of CD14, TLR2, and TLR4 expression [36–40]. Clearly, this active anti-inflammatory response of *C. canimorsus* raises a number of questions that need to be addressed: what signaling pathways are targeted and by what kind of toxin or effector? It is striking to note that other pathogens, such as *Ehrlichia chaffeensis* [39], *Francisella tularensis* [41], *Listeria monocytogenes* [42], and *Leishmania donovani* [43], also down-regulate the inflammatory cascade through p38 inhibition. Whether these targets are modulated by common mechanisms or by similar effectors is yet to be determined.

C. canimorsus is a commensal of dogs' and cats' mouths. Recent observations have shown that commensal bacteria often do not trigger an inflammatory response but instead, in some cases, have the capacity to actively turn it down. Hence, commensals may be viewed as regulatory quenchers of the inflammation, preventing the progression from the acute to the chronic state [44]. In conclusion, *C. canimorsus* possesses both passive and active mechanisms of immune evasion. It is likely that the passive mechanism involves the escape of TLR4 detection through a unique LPS structure. Although it remains unclear how *C. canimorsus* antagonizes TLR4 activation by the active mechanism, it seems to target critical players that are also manipulated by other pathogens. A better understanding of this mechanism could lead not only to the prevention of these dramatic infections but also to a better understanding of the regulation of the innate immune system.

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